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Digestive proteinases of the larger black flour beetle, *Cynaeus angustus* (Coleoptera: Tenebrionidae)

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Abstract

Digestion in the larger black flour beetle, *Cynaeus angustus* (LeConte), was studied to identify new control methods for this pest of stored grains and grain products. The physiological pH of the larval gut, as measured with extracts in water, was approximately 6.1, and the pH for optimal hydrolysis of casein by gut extracts was 6.2 when buffers were reducing. However, under non-reducing conditions, hydrolysis of casein and synthetic serine proteinase substrates was optimal in alkaline buffer. Three major proteinase activities were observed in zymograms using casein or gelatin. Caseinolytic activity of *C. angustus* gut extracts was inhibited by inhibitors that target aspartic and serine proteinase classes, with minor inhibition by a cysteine proteinase inhibitor. In particular, soybean trypsin and trypsin/chymotrypsin inhibitors were most effective in reducing the *in vitro* caseinolytic activity of gut extracts. Based on these data, further studies are suggested on the effects of dietary soybean inhibitors of serine proteinases, singly and in combination with aspartic and cysteine proteinase inhibitors, on *C. angustus* larvae. Results from these studies can be used to develop new control strategies to prevent damage to grains and stored products by *C. angustus* and similar coleopteran pests.

Keywords: *Cynaeus angustus*, digestive proteinases, control, stored products

Introduction

The larger black flour beetle, *Cynaeus angustus* (LeConte) (Coleoptera: Tenebrionidae), is a pest commonly associated with stored products throughout the USA and Canada, mainly during the summer and autumn (Dunkel *et al*., 1982). Originally the species inhabited portions of southwestern USA, from Arizona to California. By the 1940s, the geographic range of *C. angustus* extended to southern Texas, Minnesota, Canada, and east to Illinois (Krall & Decker, 1944). Although corn is the preferred food, *C. angustus* has also been found in wheat, sorghum, soybean, barley, oats, cotton gin waste, crop residues, human residences, retail stores, and is common at the base of desert yucca plants (Dunkel *et al*., 1982). More recently, infestations of *C. angustus* have been reported in cotton gin waste byproducts, and these breeding grounds can be especially problematic for homeowners (Morrison & Dunkel, 1983; McIntyre & Porter, 2004).

Cynaeus angustus has no diapause and breeds continuously under favourable temperatures, optimally 30°C (Krall & Decker, 1944). Most damage occurs during larval stages of development, typically from 46–55 days post-emergence at 30°C. *Cynaeus angustus* larvae consume more than other beetles, approaching the same level of destruction as lepidopteran pests, such as *Plodia interpunctella* (Hübner) (White & Sinha, 1987). Adults can disperse over long distances and can survive over 100 days. *Cynaeus angustus* adult consumption is also greater than other stored product pests, consuming a little more than two kernels of corn per individual (White & Sinha, 1987). The success of *C. angustus* as a cosmopolitan pest has been attributed to its survival on a variety of food sources in a wide range of environmental conditions (Sinha, 1973).

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Digestion has been studied in several other tenebrionids. Thie & Houseman (1990) reported primarily cysteine proteinase acitivty in the larval anterior midgut of *Tenebrio molitor* Linnaeus, but serine proteinase activity was prominent in the anterior midgut. A number of trypsin and chymotrypsin digestive enzymes have been described in larvae of *T. molitor* (Applebaum *et al*., 1964; Levinsky *et al*., 1977; Applebaum, 1985; Terra *et al*., 1985; Ferriera *et al*., 1990; Thie & Houseman, 1990; Terra & Cristofoletti, 1996; Cristofoletti *et al*., 2001). More recently, the major digestive trypsin and chymotrypsin enzymes were characterized in detail (Elpidina *et al*., 2005; Tsybina *et al*., 2005). Cysteine proteinases have also been isolated from *T. molitor* larval midgut (Cristofoletti *et al*., 2005). In another tenebrionid, *Tribolium castaneum* (Herbst), cysteine proteinases are the major digestive proteinases, with a small contribution by serine proteinases (Oppert *et al*., 1993, 2003). However, under dietary conditions that contain inhibitors of cysteine proteinases, serine proteinase in *T. castaneum* larvae are more important in food digestion (Oppert *et al*., 2005).

Transgenic technology has improved seed resistance to pests using microbial proteins from *Bacillus thuringiensis*, yet the control of pests with transgenic plants containing genes encoding proteinase inhibitors has been less successful, in part due to the adaptive response of insects (reviewed in Oppert, 2000). However, knowledge of the digestive physiology of insect pests can provide information to predict and prevent adaptive responses to proteinase inhibitors, as well as insights into new control technologies. Therefore, we studied *C. angustus* larval stage digestive proteinases to identify proteinase inhibitors that may be used alone or with other insecticidal control proteins as novel larvicides.

Materials and methods

Insect dissection

The *C. angustus* colony originated from beetles collected in grain bins in Concordia, Kansas in August 1993 and has been maintained in the laboratory on cracked corn. One day prior to dissection, larvae were switched to yeast-enriched (5%) flour to ensure that midguts contained food. In addition, the number of larvae per container was reduced to between 20–50 larvae per pint jar of flour to induce feeding. Dissected larvae had head capsules measuring an average of 1.00 ± 0.02 mm ($n = 15$ groups of 3–26 larvae) and weighing an average of 9.01 ± 0.39 mg ($n = 15$ groups of 3–26 larvae). Head capsules measuring 1.0 mm are indicative of the ninth instar larval stage (White & Sinha, 1987). However, the average weight of *C. angustus* larvae used in this study was considerably greater than the previously reported average of 3 mg for ninth instar larvae (White & Sinha, 1987).

Prior to dissection, larvae were placed on ice, the posterior and anterior ends were removed, and the gut was extracted through the posterior portion of the animal using forceps. Guts were immediately placed in 25 µl of ice-cold buffer A (200 mM Tris, pH 8.0, 20 mM $CaCl_2$) and frozen at −20°C. For assays, the samples were thawed, pooled, vortexed for 2 min, and centrifuged at $15{,}000 \times g$ for 5 min. The supernatant of the gut sample containing soluble proteins was used for all biochemical assays.

Analysis of gut pH

Two midgut samples were placed into separate microcentrifuge tubes, each containing 25 µl distilled deionized water. Tubes were vortexed for 2 min, centrifuged at $15{,}000 \times g$ for 5 min, and the pH was measured with a semi micro pH electrode (Microelectrodes, Inc., Bedford, New Hampshire) using a Corning 440 pH meter (Corning, New York).

Microplate proteinase assays

The procedure was adapted from a previously described microplate assay (Oppert *et al*., 1997). For pH curves, 0.20 gut equivalent (5 µl) was diluted into 85 µl of buffers of various pH, using a universal buffering system (Frugoni, 1957). Ten µl of substrate (prepared as per manufacturer's protocol) containing 0.1 µg fluorescently-labelled casein (BODIPY-TR-X casein, Molecular Probes, Eugene, Oregon) was added to each well to initiate the reaction. Samples were incubated at 37°C, and the fluorescence was measured (excitation 584; emission 620) using a Fluoroskan Ascent FL microplate reader (Labsystems, Thermo Electron Corp., Milford, Massachusetts) and corrected by subtracting readings obtained with incubations of substrate only (no enzyme). Measurements of enzyme and buffer or buffer only produced negligible fluorescence.

Where indicated, reducing conditions were achieved by adding 5 mM L-cysteine to buffers. Proteinase inhibitors/activators used in this study include [ethylenediamine] tetraacetic acid (EDTA), aprotinin, chymostatin, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), leupeptin, pepstatin, phenylmethylsulphonyl fluoride (PMSF), (L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone-HCl) (TLCK), (L-1-chloro-3-[4-tosylamido]-4-phenyl-2-butanone) (TPCK), and soybean Kunitz trypsin and Bowman-Birk trypsin-chymotrypsin inhibitors (Sigma Chemical Co., St Louis, Missouri). Inhibitors were added to pH 6.19 buffer with 5 mM L-cysteine and serially diluted 1 : 1. Gut extract was preincubated at 37°C for 30 min prior to the addition of substrate, and the remainder of the procedure was performed as described in the microplate assay with BODIPY.

To evaluate serine proteinase activities, class-specific substrates, α-benzoyl-L-arginine ρ-nitroanilide (BApNA), specific for trypsin-like proteinases, and N-succinyl-ala-ala-pro-phe ρ-nitroanilide (SAAPFpNA), specific for chymotrypsin-like proteinases, were used (Sigma). Stock substrates of BApNA (100 mg ml^{-1} in dimethyl formamide) and SAAPFpNA (100 mg ml^{-1} in dimethyl sulphoxide) were diluted 25 µl ml^{-1} in each pH buffer. Two µl containing 0.04 gut equivalents were added to 48 µl of each universal pH buffer per well. Prior to reading the plates, 50 µl of diluted substrate was added to each well (50 µg per well). Plates were incubated at 37°C for 5 min and absorbance was read at 405 nm in 15 intervals. The change in absorbance per min was calculated by KinetiCalc3 software (BIO-TEK, Winooski, Vermont). Specific activity was defined as the µmoles of nitroaniline released per min per gut equivalent (extinction coefficient of 8700 M^{-1} cm^{-1}).

Zymogram analysis

Three different types of zymograms were used to analyse gut proteinases from *C. angustus*. These included gels

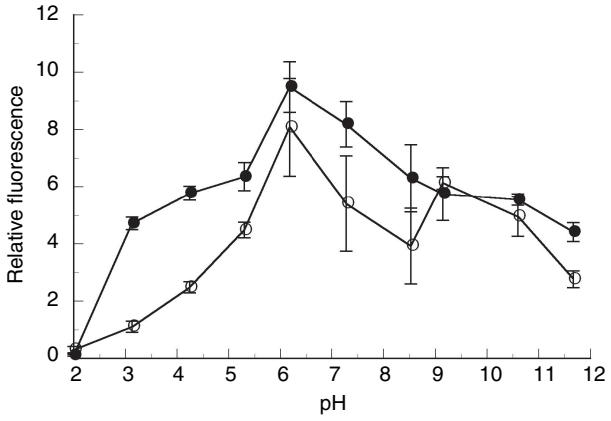

Fig. 1. Effect of pH on the caseinolytic activity of larval gut extracts of *Cynaeus angustus* when buffers contained (●) or lacked (○) a reducing reagent, L-cysteine, as indicated ($n = 3$, ±S.D.).

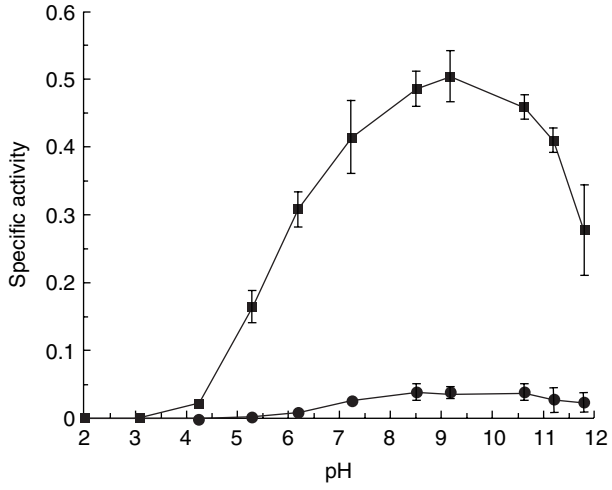

Fig. 2. Hydrolysis of class specific substrates, BApNA (●) and SAAPFpNA (■), by gut extracts from *Cynaeus angustus* ($n = 3$, ±S.D.).

impregnated with stained casein (4–16% ZBC) or gelatin (10%), or 4–12% Bis-Tris gels without substrate (Invitrogen, Carlsbad, California). Molecular mass markers were a commercial preparation (MultiMark, Invitrogen). Proteins (0.2 gut equivalents per well) were separated by electrophoresis without prior heating, and gels were incubated in zymogram developing buffer, 50 mM Tris, pH 8.0, 200 mM $NaCl_2$, 0.02% Brij 35 (Invitrogen) for 5 h. Bis-Tris gels were incubated in zymogram developing buffer with 1% casein (ICN, Aurora, Ohio). Following incubation, gels were washed with deionized water and stained with Coomassie blue.

Proteinase activity blots

Gut extracts (0.5 gut equivalents per lane) from *C. angustus* larvae were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10–20% tricine gels and compared to MultiMark molecular mass markers (Invitrogen). Gels were rinsed with deionized water, electrotransferred to nitrocellulose, incubated with ρ-nitroanilide substrates in buffer A, incubated at 37°C until yellow colour appeared, and developed as described previously (Oppert & Kramer, 1998).

Results

To determine the conditions in the gut that affect digestive proteolysis, larvae of *C. angustus* were dissected and midguts homogenized in distilled water. The average pH measured 6.1 ($n = 2$). Gut extracts were assayed for general proteolytic activity using a fluorescent casein substrate, and optimal activity was achieved in buffers around pH 6.0 in buffers containing reducing reagent (fig. 1). Two major groups of activity were observed in acidic buffers, with a slow increase of activity from around pH 3 to 5.5, and a sharp increase in activity from 5.5 to 6.2, suggesting multiple proteinase activities in this pH range. However, under non-reducing conditions, an additional peak of activity was observed in pH 9.2 buffer, indicative of serine proteinase activity.

Diagnostic substrates were tested with gut extracts, and the chymotrypsin substrate SAAPFpNA was hydrolysed to a greater degree than the trypsin substrate BApNA (fig. 2). The pH for maximal hydrolysis of SAAPFpNA was 9.1, slightly lower than the alkaline pH for maximal hydrolysis of casein in non-reducing buffers. BApNA was hydrolysed at low levels in all alkaline buffers.

Zymogram analyses of *C. angustus* gut extracts revealed the number and relative intensity of proteinases capable of hydrolysing a particular substrate. In gels containing casein, three major groups of proteinase activities were observed, with a strong activity at approximately 15 kDa, and minor caseinolytic activities at 18 and >60 kDa (fig. 3a). However, casein in zymogram gels can affect the migration of proteinases during electrophoresis. Therefore, a casein-infusion zymogram assay was also used, in which proteinases were first separated and then incubated with casein substrate in solution. In this assay, only two major activities were observed, at approximately 25 and 50 kDa (fig. 3b). While this assay represents more accurately the relative molecular masses of proteinases, it is less sensitive and was unable to detect the larger molecular mass proteinase observed in the previous zymogram. In gelatin zymograms, three major proteinases were detected, corresponding to molecular mass markers at approximately 25, 50, and 100 kDa, as well as gelatinase activity remaining in the stacking gel.

An activity blot assay, using the substrate SAAPFpNA, demonstrated a strong chymotrypsin-like activity at approximately 25 kDa (fig. 4), similar to the faster migrating activities observed in zymograms (fig. 3). A minor chymotrypsin-like activity was detected at >100 kDa and was similar to the slower migrating activity in zymograms. No activity was detectable in activity blots with the substrate BApNA (data not shown).

Finally, microplate assays with class specific inhibitors were used to further characterize the digestive proteinases of *C. angustus*, as well as identify candidate inhibitors for control of *C. angustus* damage to stored grains. The most effective inhibitors were from soybean, including inhibitors

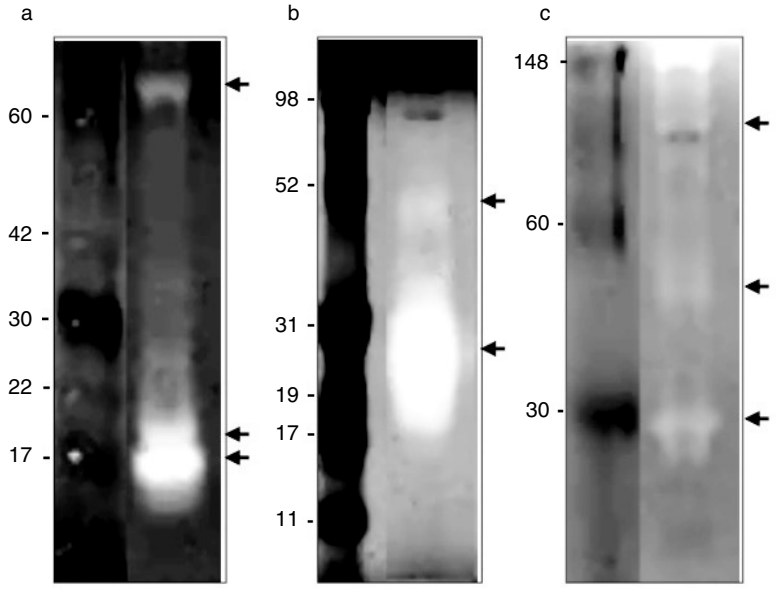

Fig. 3. Zymogram analysis of caseinolytic activities (a and b) or gelatinase activity (c) of gut extracts from *Cynaeus angustus*. Zymograms a and c have casein and gelatin, respectively, impregnated in the gel, whereas zymogram b has no casein in the gel during electrophoresis, but instead is incubated in a casein solution post electrophoresis. Molecular mass markers are indicated on the left.

of chymotrypsin-trypsin (Bowman-Birk) and trypsin (Kunitz), with IC_{50} values of 0.541 and 0.569 μM, respectively (fig. 5). Pepstatin was a moderately effective inhibitor, with an IC_{50} of 6.56 μM, suggesting that the acidic caseinolytic activity may be due at least in part to aspartic proteinases. Other effective inhibitors included leupeptin, a serine/cysteine proteinase inhibitor and aprotinin, a serine proteinase inhibitor, with IC_{50} values of 4.12 and 10.0 μM, respectively. PMSF, a more non-specific serine proteinase inhibitor, was approximately 10-fold less active, with an IC_{50} of 111.0 μM. Chymostatin and TLCK, inhibitors of chymotrypsin, and E-64, which inhibits mostly cysteine and sometimes serine proteinases, demonstrated slight inhibition of approximately 20–40% when higher levels of inhibitors were used.

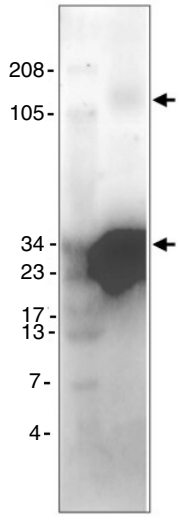

Fig. 4. Activity blot analysis of gut extract from *Cynaeus angustus*, using the substrate SAAPFpNA. Molecular mass markers are indicated on the left.

Discussion

The results from *in vitro* analysis of gut proteinases and synthetic substrates indicated that *C. angustus* larvae have digestive proteinases active in acidic and alkaline pH buffers. Peaks of activity in acidic buffers may be due to aspartic proteinases, but the stimulation of activity by thiol reagents in these buffers may also indicate the presence of cysteine proteinases. Based on the hydrolysis of a chymotrypsin substrate by crude gut extracts, chymotrypsin-like proteinases are predicted to be important enzymes for *C. angustus* food digestion, with minor activity by trypsin-like proteinases. Combined, these data suggested that acidic, cysteine, and serine proteinases contribute to digestion in larval *C. angustus*.

Electrophoretic analysis of proteins in *C. angustus* gut extracts indicated at least three major groups of enzyme activities. A faster-migrating activity resolved into two distinct areas of hydrolysis in the casein zymogram and was a larger smear of activity in the casein infusion and activity blot assays. These activities are likely to represent several chymotrypsin-like *C. angustus* proteinases. In addition, another larger molecular mass chymotrypsin activity was observed in activity blots that may correspond to the larger molecular mass caseinolytic and gelatinase activities. These larger molecular mass activities may represent lipid-associated proteinases that have been previously described (Bolognesi *et al*., 2001). Differences in the relative molecular masses can be attributed to the different gel gradients, differential substrate hydrolysis, as well as the presence or absence of substrate in the gel. A minor proteinase

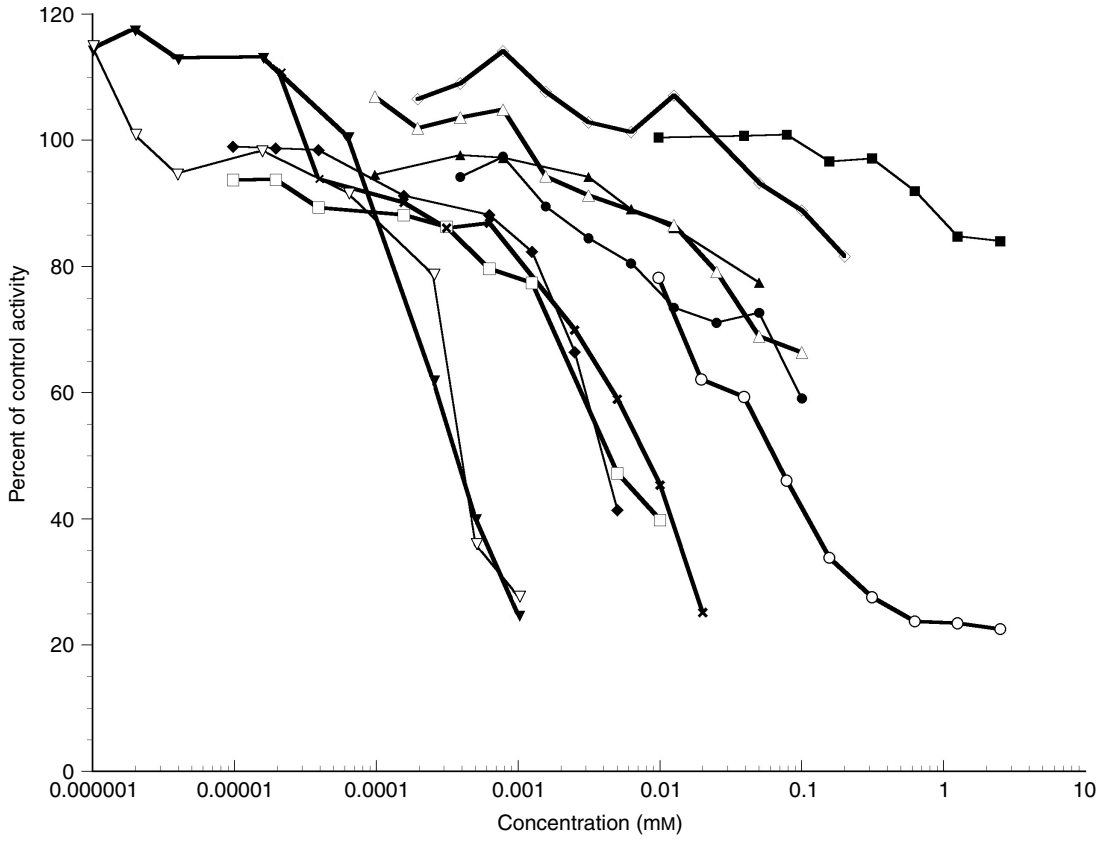

Fig. 5. Inhibition of the caseinolytic activity of gut extracts from *Cynaeus angustus* by inhibitors at different concentrations. ■, EDTA; ●, chymotrypsin; ▲, E-64; ◆, leupeptin; ▼, SBTI; □, pepstatin; ○, PMSF; △, TLCK; ◇, TPCK; ▽, SKTI; ×, aprotonin.

activity with a molecular mass of around 50 kDa was more pronounced in the casein infusion zymogram but also was observed in the gelatinase zymogram. This activity was not observed in the chymotrypsin activity blots and is probably a different type of proteinase.

Overall, inhibitors of serine proteinases were effective in reducing the caseinolytic activity of enzymes extracted from *C. angustus* larvae. The most effective inhibitors were from soybean and microbial sources. Inhibition by pepstatin is diagnostic for aspartic proteinase activity, and about 60% of the total caseinolytic activity of *C. angustus* larval gut proteinases was inhibited by 10 μM pepstatin. E-64 inhibition is usually an prediction of cysteine proteinase activity, but at the highest concentration of E-64 tested (100 μM), only a 20% loss of activity was observed. Inhibition by E-64 did not increase in reducing acidic buffers (data not shown), and therefore it was concluded that cysteine proteinases, if present, constitute only a minor portion of the total proteolytic activity in *C. angustus* larvae.

The combined data indicated that *C. angustus* larvae have a complex system of protein digestion, incorporating proteinases from serine, cysteine and aspartic classes. This may be the result of adaptation to feeding on diverse food sources. It also suggests that the control of *C. angustus* larvae through proteinase inhibitor technology will be problematic. Testing of inhibitors *in vivo*, individually and in combination, will determine the efficacy of inhibitor-containing diets to reduce the growth and development of *C. angustus* larvae. Based on the results in this study, candidate inhibitors include those from soybean, as well as proteinaceous inhibitors of aspartic and cysteine proteinases. Combinations of inhibitors may be particularly effective in increasing mortality, as has been found with another tenebrionid, *T. castaneum* (Oppert *et al*., 1993, 2003, 2004). Inhibitor combinations may also prevent the adaptive response to individual inhibitors, as was documented with *T. castaneum* larvae (Oppert *et al*., 2005). These studies will provide information on the feasibility of using proteinase inhibitors, either through gene or protein biopesticide technology, to reduce seed damage by *C. angustus*.

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References

Applebaum, S.W. (1985) Biochemistry of digestion. pp. 279–311 *in* Kerkut, G.A. & Gilbert, L.I. (*Eds*) *Comprehensive insect physiology, biochemistry, and pharmacology*. Oxford, Pergamon Press.

Applebaum, S.W., Birk, Y., Harpaz, I. & Bondi, A. (1964) Comparative studies on proteolytic enzymes of *Tenebrio molitor* L. *Comparative Biochemistry and Physiology* **11**, 85–103.

Bolognesi, R., Ribeiro, A.F., Terra, W.R. & Ferreira, C. (2001) The peritrophic membrane of *Spodoptera frugiperda*: secretion of peritrophins and role in immobilization and recycling digestive enzymes. *Archives of Insect Biochemistry and Physiology* **47**, 62–75.

Cristofoletti, P.T., Ribeiro, A.F. & Terra, W.R. (2001) Apocrine secretion of amylase and exocytosis of trypsin along the midgut of *Tenebrio molitor* larvae. *Journal of Insect Physiology* **47**, 143–155.

Cristofoletti, P.T., Ribeiro, A.F. & Terra, W.R. (2005) The cathepsin L-like proteinases from the midgut of *Tenebrio molitor* larvae: sequence, properties, immunocytochemical localization and function. *Insect Biochemistry and Molecular Biology* **35**, 883–901.

Dunkel, F.V., Barak, A.V. & Harein, P.K. (1982) Geographical distribution of *Cynaeus angustus* (LeConte) (Coleoptera: Tenebrionidae) and its association with stored products. *Journal of Biogeography* **9**, 345–352.

Elpidina, E.N., Tsybina, T.A., Dunaevsky, Y.E., Belozersky, M.A., Zhuzhikov, D.P. & Oppert, B. (2005) A chymotrypsin-like proteinase from the midgut of *Tenebrio molitor* larvae. *Biochimie* **87**, 771–779.

Ferreira, C., Bellinello, G.L., Ribeiro, A.F. & Terra, W.R. (1990) Digestive enzymes associated with the glycocalyx, microvillar membranes and secretory vesicles from midgut cells of *Tenebrio molitor* larvae. *Insect Biochemistry* **20**, 839–847.

Frugoni, J.A.C. (1957) Tampone universale di Britton e Robinson a forza ionica costante. *Gazzetta Chimica Italiana* **87**, 403–407.

Krall, J.L & Decker, G.C. (1944) The biology of *Cynaeus angustus* Lec., a new stored grain pest. *Iowa State College Journal of Science* **20**, 385–402.

Levinsky, H., Birk, Y. & Applebaum, S.W. (1977) Isolation and characterization of a new trypsin-like enzyme from *Tenebrio molitor* L. larvae. *International Journal of Peptide and Protein Research* **10**, 252–264.

McIntyre, N.E. & Porter, P. (2004) Field trial of diatomaceous earth in cotton gin trash against the larger black flour beetle, *Cynaeus angustus* (Coleoptera: Tenebrionidae). *Journal of Economic Entomology* **97**, 588–592.

Morrison, W.P. & Dunkel, F.V. (1983) *Cynaeus angustus* (LeConte) as a nuisance pest associated with cotton gin waste. *Southwestern Entomologist* **8**, 90–93.

Oppert, B. (2000) Transgenic plants expressing enzyme inhibitors and the prospects for biopesticide development. pp. 83–95 *in* Koul, O. & Dhaliwal, G.S. (*Eds*) *Phytochemical biopesticides*. The Netherlands, Harwood Academic.

Oppert, B. & Kramer, K.J. (1998) Economical proteinase activity blot. *American Biotechnology Laboratory* **16**, 20.

Oppert, B., Morgan, T.D., Culbertson, C. & Kramer, K.J. (1993) Dietary mixtures of cysteine and serine proteinase inhibitors exhibit synergistic toxicity toward the red flour beetle, *Tribolium castaneum*. *Comparative Biochemistry and Physiology* **105C**, 379–385.

Oppert, B., Kramer, K.J. & McGaughey, W.H. (1997) Rapid microplate assay of proteinase mixtures. *BioTechniques* **23**, 70–72.

Oppert, B., Morgan, T.D., Hartzer, K., Lenarcic, B., Galesa, K., Brzin, J., Turk, V., Yoza, K., Ohtsubo, K. & Kramer, K.J. (2003) Effects of proteinase inhibitors on growth and digestive proteolysis of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Comparative Biochemistry and Physiology* **134C**, 481–490.

Oppert, B., Morgan, T.D. & Kramer, K.J. (2004) Inhibitor strategies to control coleopteran pests. vol. 1275, pp. 149–156 *in* Morris, S. & Vosloo, A. (*Eds*) *International Congress Series, Animals and Environments. Proceedings of the Third International Conference of Comparative Physiology and Biochemistry*. Amsterdam, Elsevier.

Oppert, B., Morgan, T.D., Hartzer, K. & Kramer, K.J. (2005) Compensatory proteolytic responses to dietary proteinase inhibitors in the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Comparative Biochemistry and Physiology, Part C*, in press.

Sinha, R.N. (1973) Ecology of storage. *Annals of Technology of Agriculture* **22**, 351–369.

Terra, W.R. & Cristofoletti, P.T. (1996) Midgut proteinases in three divergent species of Coleoptera. *Comparative Biochemistry and Physiology* **113B**, 725–730.

Terra, W.R., Ferreira, C. & Bastos, F. (1985) Phylogenetic consideration of insect digestion. Disaccharidases and the spatial organization of digestion in the *Tenebrio molitor* larvae. *Insect Biochemistry* **15**, 443–449.

Thie, N.M.R. & Houseman, J.G. (1990) Cysteine and serine proteolytic activities in larval midgut of yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). *Insect Biochemistry* **20**, 741–744.

Tsybina, T.A., Dunaevsky, Y.E., Belozersky, M.A., Zhuzhikov, D.P., Oppert, B. & Elpidina, E.N. (2005) Digestive proteinases of yellow mealworm (*Tenebrio molitor*) larvae: purification and characterization of a trypsin-like proteinase. *Biochemistry (Moscow)* **70**, 300–305.

White, N.D.G. & Sinha, R.N. (1987) Bioenergetics of *Cynaeus angustus* (Coleoptera: Tenebrionidae) feeding on stored corn. *Annals of the Entomological Society of America* **80**, 184–190.

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